FEBS 26183 FEBS Letters 521 (2002) 157–164

Protein kinase A balances the growth factor induced Ras/ERK signaling

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Received 25 February 2002; revised 10 April 2002; accepted 23 April 2002

First published online 29 May 2002

Edited by Richard Marais

Abstract Protein kinase A (PKA) has been proposed to regulate the signal transduction through the Ras/extracellular-regulated kinase (ERK) pathway. Here we demonstrate that when the PKA activity was inhibited prior to growth factor stimulus the signal flow through the Ras/ERK pathway was significantly increased. Furthermore, the data indicated that this PKA-mediated regulation was simultaneously targeted to the upstream kinase Raf-1 and to the ERK-specific phosphatase mitogen-activated protein kinase phosphatase-1 (MKP-1). Moreover, our data suggested that the level of PKA activity determined the transcription rate of *mkp-1* gene, whereas the Ras/ERK signal was required to protect the MKP-1 protein against degradation. These results point to a tight regulatory relationship between PKA and the growth factor signaling, and further suggest an important role for basal PKA activity in such regulation. We propose that PKA adjusts the activity of the Ras/ERK pathway and maintains it within a physiologically appropriate level. © **2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Protein kinase A; Fibroblast growth factor-2; Growth factor; Extracellular-regulated kinase; Raf-1; Mitogen-activated protein kinase phosphatase-1

1. Introduction

Binding of growth factors to the transmembrane receptor leads to activation of intracellular signaling pathways. The main pathway transporting these signals is the Ras/extracellular-regulated kinase (ERK) pathway. Depending on the nature of the extracellular stimulus, activation of the Ras/ERK pathway may contribute to cell growth, differentiation or survival. Tailored responses to distinct extracellular stimuli are presumably generated by integration of different signals in the cell. Such integration comprises, at least in part, a firm control of signal magnitude and duration [1–4]. The molecular mechanisms of such regulation are not completely understood but likely involve cross-talk between different mitogen-activated protein (MAP) kinase cascades as well as protein kinases of other signaling pathways such as protein kinase A (PKA) and protein kinase C [5,6].

Recent studies suggest that cAMP and PKA participate in the regulation of the growth factor signaling [7–10]. The main molecular mediators of such regulation are the Raf-1 and B-Raf serine/threonine kinases [11,12]. In most cell types PKA down-regulates the Ras/ERK signaling by interfering with the Ras-mediated activation of Raf-1 [11,13–16]. However, in melanocytes and in PC12 cells an increase in the cAMP concentration activates the Ras/ERK pathway through the small GTP binding protein Rap-1 and/or B-Raf [17–21].

The kinase intermediates of the Ras/ERK pathway, such as ERK1 and ERK2, are regulated by phosphorylation and dephosphorylation of specific amino acid residues [22–28]. The family of dual specific MAP kinase phosphatases (MKPs), able to dephosphorylate both threonine and tyrosine residues, is an important phosphatase family involved in the direct down-regulation of MAPK signaling. According to their pattern of transcriptional regulation and cellular localization, MKPs are divided into two categories: nuclear proteins encoded by immediate early genes (e.g. MKP-1 and MKP-2) and cytosolic proteins encoded by delayed genes (e.g. MKP-3/Pyst1). Consequently, MKP-1 and MKP-2 are the most important early regulators of mitogen-induced ERK activity. The abundance of MKP proteins is controlled by the transcriptional activation of the gene and by the ubiquitin-directed degradation of the protein product [29–33]. MKP-1, MKP-2 and MKP-3 are transiently expressed following activation of the Ras/ERK pathway, suggesting a feedback loop to regulate ERK activity [34–40]. MKP-1 and MKP-2 dephosphorylate all mitogen-activated kinases albeit their substrate preferences depend on the cell model studied, whereas MKP-3 is specific for ERK1 and ERK2 [41–46]. PKA controls the function of several phosphatases able to dephosphorylate various components of the Ras/ERK pathway. Such phosphatases include serine/threonine phosphatase PP1 and tyrosine phosphatases PTP-SL and HePTP [47–49]. Moreover, PKA directly regulates the transcription of *mkp-1* gene via two cAMP response elements (CRE) located in its promoter region [50–52].

Although PKA is believed to have a critical role in the growth factor-induced Ras/ERK signaling, the mechanisms and physiological significance of such regulation are incompletely understood. Whereas accumulating evidence suggests that experimental over-activation of PKA down-regulates the Ras/ERK signaling, the role of basal PKA activity in the regulation of growth factor signaling remains unknown. We

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Abbreviations: FGF-2, fibroblast growth factor-2; EGF, epidermal growth factor; PKA, protein kinase A; ERK, extracellular-regulated kinase; H89, (*N*-[2-(*p*-bromocinnamylamino)-ethyl]-5-isoquinolinesulfonamide); 8-BrcAMP (8-bromoadenosine 3′,5′-cyclic monophosphate); MKP-1, mitogen-activated protein kinase phosphatase-1; DMEM, Dulbecco's modified Eagle's medium; 4-OHT, 4-hydroxytamoxifen; GST, glutathione *S*-transferase

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PII: S0014-5793(02)02864-8

have previously shown that in NIH3T3 fibroblasts active PKA is required for the fibroblast growth factor-2 (FGF-2)-mediated activation of an AP-1 driven gene transcription [53,54]. In this paper, we demonstrate that the down-regulation of PKA activity allows dramatic increase in the signal transduction through the Ras/ERK pathway in response to growth factor stimuli, showing that a strict control over the PKA activity is required to maintain the proper signal flow through this pathway. Our data indicate that PKA adjusts the magnitude and duration of growth factor signals by controlling the catalytic activity of Raf-1 and the expression of MKP-1. We further elucidated mechanisms by which PKA and growth factors co-operatively regulated the expression and function of MKP-1. In conclusion, our data suggest an important role for controlled PKA activity in the regulation of growth factor signaling.

2. Materials and methods

2.1. Materials

H89 (*N*-[2-(*p*-bromocinnamylamino)-ethyl]-5-isoquinolinesulfonamide) (used at 10 μM), 8-BrcAMP (8-bromoadenosine 3′,5′-cyclic monophosphate) (used at 500 μM), anisomycin, PD98059, cycloheximide and MG132 were purchased from Calbiochem. Basic FGF (FGF-2) and epidermal growth factor (EGF) were from PeproTech (Rocky Hill, NJ, USA) and were used at the concentration of 10 ng/ml. Protein-G Sepharose® Fast Flow and glutathione-Sepharose® were from Pharmacia Biotech. The monoclonal antibody for activated ERK1 and ERK2, myelin basic protein (MBP) and 4-hydroxytamoxifen (4-OHT) were purchased from Sigma. Agarose-conjugated Raf-1 (C-12:sc-133Ac), Raf-1 (C-12), MKP-1 (V-15), MKP-3 (C-20) and ERK2 (K-23) antibodies and MEK-1 (FL-1) protein were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-p38 and anti-phospho-p38 antibodies were from New England Biolabs (UK), and MKP-2 antibody from Transduction Laboratories (Lexington, KY, USA). Anti-glutathione *S*-transferase (GST) antibody was from Upstate Biotechnology (USA).

2.2. Cell culture and transfections

The mouse fibroblast cells (NIH3T3) were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS) and 1% (w/v) penicillin and streptomycin in humidified 95% air, 5% CO_2 at 37°C. When the cultures reached ~80% confluence cells were serum-starved with DMEM supplemented with 1% carboxymethyl-Sepharose-eluted FCS for 48 h and treated with growth factors and chemicals. Plasmid DNA was transfected to the cells by the calcium-phosphate precipitation method. Stably transfected cells were produced by simultaneous introduction of the pBGS plasmid and a 10× molar excess of pEBG-ERK2 plasmid (kindly provided by Dr. Michyuki Matsuda) and subsequent selection with G418 (500 μg/ml). The cDNA encoding ΔRaf-1:ER[DD] (a generous gift by Dr. Martin McMahon) was cloned into the pCDNA6 vector and transfected cells were selected on the basis of their resistance to blasticidin (1 μg/ml).

2.3. Northern analysis

To analyze transcriptional induction of *mkp-1* gene 500 000 cells were plated on 10 cm dishes and after 2–3 days cells were serum-starved and treated as indicated. Total RNA was isolated with RNA-Zol™ B (TEL-TEST, Inc., USA), run on 1% agarose gels, and transferred to a Hybond-N nylon membrane (Amersham Pharmacia). The membranes were hybridized with random-primed cDNA of the *mkp-1* gene.

2.4. Western analysis

For the analysis of ERK phosphorylation and phosphatase expression, 250 000 cells were plated on 30 mm dishes. After 2–3 days, cells were serum-starved and subjected to various treatments. Subsequently, the cells were washed once with cold phosphate-buffered saline (PBS), solubilized in 150 μl of Laemmli-SDS buffer and sonicated to shear the chromosomal DNA. The lysates were run on 12% SDS–PAGE and transferred to nitrocellulose membrane (Schleicher&Schuell, Germany). The membrane was incubated overnight at 4°C under a gentle rotation in 10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.1% Tween-20 and 5% (w/v) non-fat dry milk containing the specific antibodies. The primary antibody against active ERKs was diluted 1:10 000, whereas the anti-ERK2, anti-phospho-p38, anti-p38 antibodies were diluted 1:4000 and antibodies for specific phosphatases 1:500. The specific bands were detected using the ECL chemiluminescence detection method (Amersham) by exposure on X-ray films. To study the loading of the samples, the membranes were stripped with 0.1 M glycine (pH 2.5) (3×5 min) and washed briefly with 10 mM Tris–HCl pH 8.0, 150 mM NaCl and 0.1% Tween-20, followed by blocking and immunodetection with anti-p38 or anti-ERK2 antibodies.

2.5. Immunofluorescent staining

For immunofluorescent detection of dually phosphorylated ERK1 and ERK2 proteins, 5000 cells were plated on plastic coverslips (Amersham Pharmacia). After 2 days cells were serum-starved and treated as indicated. Cells were washed twice with cold PBS and fixed with 10% paraformaldehyde in PBS at room temperature for 15 min followed by 10 min in methanol at −20°C. The cells were then incubated with antibodies against active ERKs (1:500 in PBS containing 1% bovine serum albumin) for 16 h at 4°C under gentle shaking, washed three times with PBS and incubated with FITC-conjugated secondary antibody for 1 h in the dark at room temperature. The stainings were studied using fluorescence microscopy (Leica DMR).

2.6. ERK2 immunocomplex assay

For ERK assays 500 000 cells were plated on 10 cm dishes and after 2–3 days, cells were serum-starved and treated as indicated. Cells were washed with cold PBS and lysed in cold RIPA buffer (1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM EGTA,) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 100 U aprotinin, 10 mM leupeptin, 1 mM pepstatin and 1 mM antipain) and phosphatase inhibitors (20 mM NaF and 1 mM $NaVO_4$). The lysates were cleared by centrifugation (10 000×*g* for 10 min at 4°C). ERK2 was immunoprecipitated with 2 μg of the monoclonal anti-ERK2 antibody (2 h at 4°C) and immunocomplexes were collected by incubating with protein-G Sepharose at 4°C for 2 h. The immunoprecipitates were washed five times with cold RIPA buffer and two times with cold ERK kinase buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$) and assayed for ERK2 activity by incubating with 30 μg of MBP and 2.5 μCi of [γ-^{32}P]ATP in 30 μl of kinase buffer supplemented with 25 μM ATP at 30°C for 30 min, after which the reactions were terminated by boiling in SDS sample buffer. The samples were run on 12% SDS–PAGE, and the gels were dried and exposed on X-ray films. The amount of immunoprecipitated ERK2 was analyzed by Western blotting with anti-ERK2 antibody.

2.7. Raf-1 immunocomplex assay

For Raf-1 assays 500 000 cells were plated on 10 cm dishes and after 2–3 days, cells were serum-starved and treated as indicated. Cells were washed with cold PBS and lysed in cold HEB buffer (25 mM HEPES, pH 7.5, 10% glycerol, 5 mM EDTA, 150 mM NaCl, 0.1% β-mercaptoethanol, 1% Triton X-100,) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 100 U aprotinin, 10 mM leupeptin, 1 mM pepstatin and 1 mM antipain) and phosphatase inhibitors (20 mM Na-orthopyrophosphate, 50 mM NaF, 1 mM $NaVO_4$ and 1 mM benzamidine). The lysates were cleared by centrifugation (10 000×*g*) for 15 min at 4°C. The supernatants were assayed for protein concentration using Bio-Rad *DC* Protein Assay, and 500 μg of protein was subjected to immunoprecipitation of Raf-1 proteins overnight at 4°C using 10 μg of agarose-conjugated Raf-1 antibody (C-12:sc-133Ac). The immunoprecipitates were washed once with ice cold HEB buffer and once with ice cold LiCl buffer (500 mM LiCl, 100 mM Tris, pH 7.6, 0.1% Triton X-100), and twice with ice cold kinase buffer (20 mM HEPES, pH 7.5, 20 mM $MgCl_2$, 0.1% β-mercaptoethanol) and subjected to Raf-1 activity assay involving incubation with 2 μg of purified MEK-1 and 2.5 μCi of [γ-^{32}P]ATP in 30 μl of kinase buffer supplemented with 25 μM ATP. The mixtures were incubated at 30°C for 30 min and the reactions were terminated by boiling in SDS-gel sample buffer. The samples were run on the 12% SDS–PAGE, the gels were dried and exposed

on X-ray films. Rabbit anti-Raf-1 antibody was used to verify the equal amount of immunoprecipitated Raf-1 in the samples.

2.8. GST-pulldown assay

For GST-pulldown assays 500 000 cells expressing GST-ERK2 were plated on 10 cm dishes, and after 2–3 days cells were serum-starved and treated as indicated. Cells were washed with cold PBS and lysed in GST-pulldown buffer (20 mM HEPES pH 8.0, 2 mM EDTA, 1% Triton X-100, 10% glycerol, 50 mM β-glycerophosphate, 1 mM $NaVO_4$, 25 mM NaF) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 100 U aprotinin, 10 mM leupeptin, 1 mM pepstatin and 1 mM antipain). The lysates were cleared by centrifugation (10 000 × *g*) for 10 min at 4°C. The supernatants were measured for protein concentrations using Bio-Rad *DC* Protein Assay, combined with 50% glutathione-Sepharose slurry (40 μl slurry/800 μg protein) and incubated under mixing for 2 h at 4°C. The beads were washed three times with the GST-pulldown buffer after which the bound proteins were recovered by elution with 40 μl of 2 × SDS buffer. The GST-ERK2-bound MKP-1 was analyzed by Western-blotting.

3. Results

3.1. Inhibition of PKA activity increases growth factor-induced ERK activity

To study how PKA regulates the activation of the Ras/ERK pathway, we first followed the effects of modulation of PKA activity on the growth factor-induced phosphorylation of ERKs. Pre-treatment of the cells with the PKA inhibitor H89 substantially increased and prolonged the phosphorylation, which remained detectable as long as 9 h from the start of the FGF-2 induction. By contrast, when the cells were pre-treated with 8-BrcAMP, the level of ERK phosphorylation was significantly decreased (Fig. 1A, left panel). With EGF phosphorylation of ERKs it was more transient than that induced by FGF-2 and decreased significantly 30 min after the initiation of the EGF stimuli. Inhibition of PKA with H89 clearly increased and prolonged the ERK phosphorylation, although the response was somewhat less prominent than that seen with FGF-2, whereas stimulation of PKA decreased ERK phosphorylation (Fig. 1A, right panel). In the absence of growth factor, H89 or 8-BrcAMP did not induce phosphorylation of ERKs (data not shown). To ensure that the down-regulatory effect of 8-BrcAMP on the growth factor-induced activation of ERKs was mediated by PKA, we pre-treated cells with 8-BrcAMP for 15 min, followed by a 15 min H89 treatment prior to growth factor induction (lower panels in Fig. 1A). We further investigated the effects of PKA modulation on the catalytic activity of ERK2 by measuring the ability of immunoprecipitated ERK2 to phosphorylate MBP. Inhibition of PKA by H89 prior to FGF-2 induction markedly increased the catalytic activity of ERK2, whereas treatment with 8-BrcAMP decreased the activity (Fig. 1B).

To study whether PKA also influenced other growth factor-regulated MAPK pathways, we investigated the effect of PKA modulation on the p38 pathway using antibodies against phospho-p38. Whereas FGF-2, EGF and anisomycin were all able to induce phosphorylation of p38, the phosphorylation was not significantly altered by modulation of PKA activity (Fig. 1C). These data suggest that PKA may specifically control the Ras/ERK pathway.

3.2. Inhibition of PKA activity increases FGF-2-induced nuclear localization of activated ERKs

Activated ERKs are dimerized and translocated to the nucleus where they phosphorylate specific substrates, including transcription factor Elk-1, leading to activation-specific genes such as *c-Fos* [55–57]. To further study how PKA affects the activity of ERKs, we followed the nuclear entry and residence of activated ERKs in cells treated with FGF-2 and the modulators of PKA activity. Immunofluorescent studies with anti-phospho-ERK1/2 antibodies revealed that inhibition of PKA activity increased the nuclear entry and prolonged the nuclear residence of activated ERKs (Fig. 2I–L). By contrast, when the cells were pre-treated with 8-BrcAMP the nuclear staining was almost completely abolished (Fig. 2M–P). These results are in good agreement with the observations of how PKA regulated the FGF-2-induced phosphorylation and catalytic activity of ERKs. Our results likely represent PKA dependent changes in FGF-2-induced ERK activation. However, we note

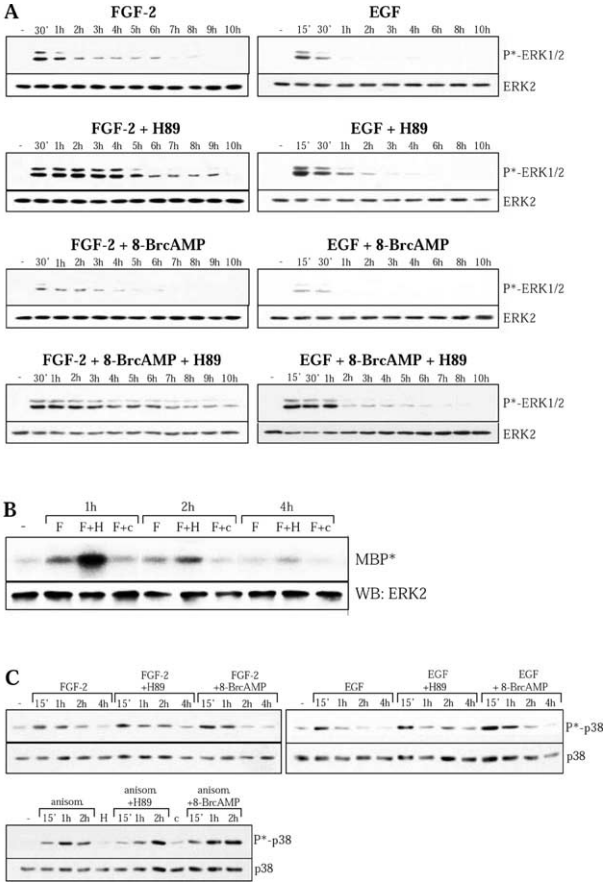

Fig. 1. PKA regulates growth factor-induced activity of ERKs. NIH3T3 cells were serum-starved and treated with PKA inhibitor H89 or PKA activator 8-BrcAMP for 30 min prior to stimulation with (A) FGF-2 or EGF. Phosphorylation of ERKs was detected by anti-phospho-ERK1/2 immunoblot of total cell lysates (P*-ERK1/2). The loading of the samples was studied by immunoblotting with anti-ERK2 antibodies. The immunoblots representing the different treatments were exposed on the same film for comparability of the data. B: Autoradiogram showing phosphorylation of MBP by immunoprecipitated ERK2. The amount of immunoprecipitated ERK2 protein in the samples was studied with anti-ERK2 immunoblot. C: Cells were treated with FGF-2, EGF or anisomycin (anisom; 2 μg/ml) alone or in combination with PKA modulators. Phosphorylation of p38 was assessed by immunoblotting with antibodies against activated p38 (P*-p38). All these experiments were repeated at least three times with similar results.

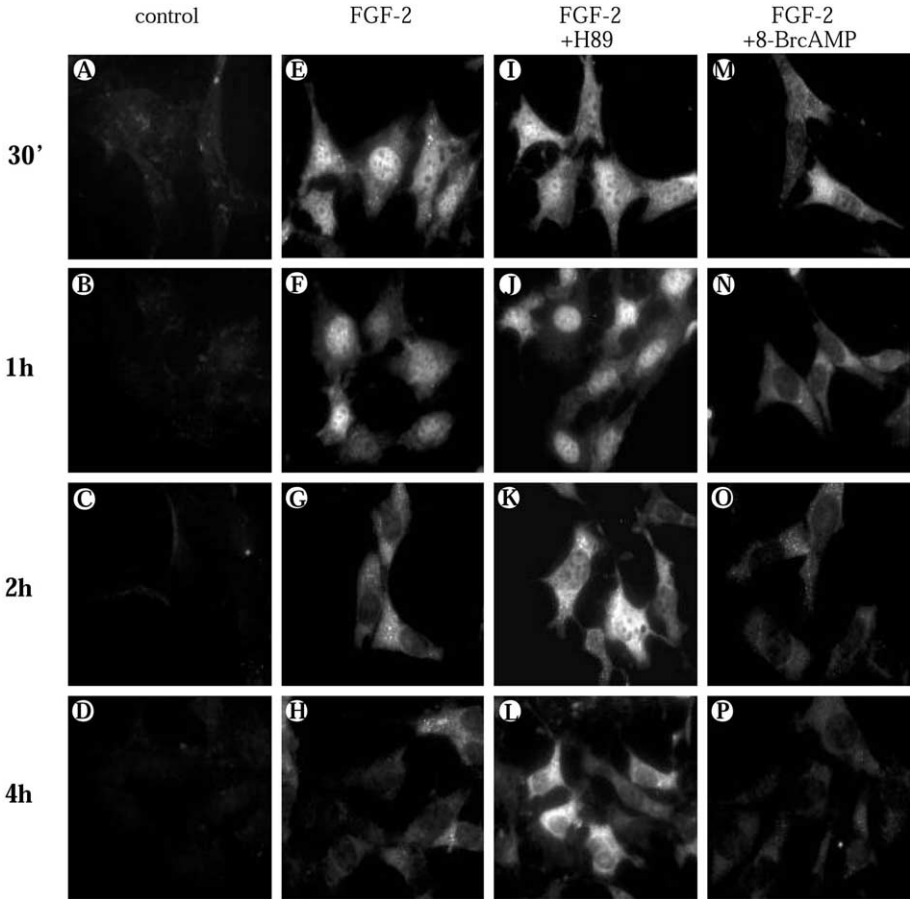

Fig. 2. Effect of PKA activity on nuclear entry and residence of activated ERKs. Cells were grown on class coverslips, serum-starved and left untreated (A–D), treated with FGF-2 alone (E–H) or in combination with modulators of PKA activity (I–P). After the indicated time points the cells were fixed and stained with a monoclonal antibody against activated ERKs. The localization of activated ERKs was analyzed using a fluorescence microscope (Leica 4D). The experiment was repeated three times with similar results, representative pictures are shown.

that PKA has been proposed to regulate the nuclear entry of ERKs in neuronal cells [58] and serum response factor in fibroblasts [59], raising an alternative possibility that deregulated PKA activity would affect the nuclear transport of activated ERKs in a more direct fashion.

3.3. Inhibition of PKA activity increases growth factor-induced activity of Raf-1

cAMP and PKA have been shown to activate the Ras/ERK pathway via B-Raf, whereas inhibition is mediated through PKA-specific phosphorylation of Raf-1 proteins [7,15,16,18, 60–62]. Since previous reports and our studies (data not shown) suggest that cAMP and PKA do not activate the Ras/ERK pathway in NIH3T3 fibroblasts, we studied the effects of PKA modulation on the catalytic activity of Raf-1. Raf-1 was immunoprecipitated from cells treated with FGF-2, EGF and PKA modulators, and analyzed for its ability to phosphorylate MEK-1. Inhibition of PKA activity prior to FGF-2 stimulation significantly increased and prolonged the Raf-1 activity, whereas treatment of cells with 8-BrcAMP almost completely abolished Raf-1 activation (Fig. 3A). PKA also regulated the Raf-1 activation induced by EGF, although the maximal effect of PKA inhibition was seen earlier than in cells stimulated with FGF-2 (Fig. 3B). These results indicate that a basal PKA activity may be required to adjust the activation level of Raf-1.

3.4. Regulation of MKP-1 expression by PKA and growth factors

Given the findings that MKP-1 and MKP-2 are encoded by growth factor-responsive immediate early genes [30,35,63] and that cAMP regulates the transcription of *mkp-1* gene [50–52],

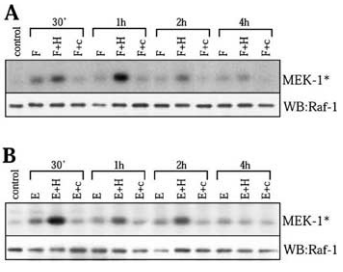

Fig. 3. Inhibition of PKA activity increases the growth factor-induced activation of Raf-1. Autoradiograms demonstrating phosphorylation of MEK-1 by immunoprecipitated Raf-1. Cells were serum-starved and treated with (A) FGF-2 or (B) EGF alone or in combination with H89 (H = H89) or 8-BrcAMP (c = 8-BrcAMP). After indicated time points the cells were lysed and the lysates were subjected to immunoprecipitation with agarose-conjugated Raf-1 antibodies followed by kinase reactions using MEK-1 as substrate. The amount of immunoprecipitated Raf-1 in the samples was analyzed by anti-Raf-1 immunoblot. MEK-1*, phosphorylated MEK-1; WB, Western blot. The experiment was repeated three times with similar results.

we investigated how PKA affects the growth factor-induced expression of these two phosphatases. In addition, we studied the expression of MKP-3, although it is not encoded by an immediate early gene [64].

Immunoblotting with anti-MKP antibodies (Fig. 4A) indicated that FGF-2 rapidly induced the expression of MKP-1. Pre-treatment of cells with H89 decreased and delayed the expression of MKP-1, such that the protein was detectable not until 2 h after the start of the FGF-2 induction. Conversely, when PKA was activated with 8-BrcAMP prior to FGF-2 stimuli, the expression of MKP-1 increased significantly. The expression of MKP-2 appeared very low and was not influenced by FGF-2 or modulation of PKA activity, whereas the expression of MKP-3 was increased in response to FGF-2, although the induction was clearly delayed. Interestingly, the MKP-3 expression appeared to be regulated by PKA, but it differed from that seen for MKP-1, since both stimulation and inhibition of PKA activity decreased the expression. In EGF-stimulated cells, PKA regulated the expression of MKPs in a largely similar fashion than in FGF-2-treated cells, although the expression of MKP-1 following the PKA activation was more transient (Fig. 4B).

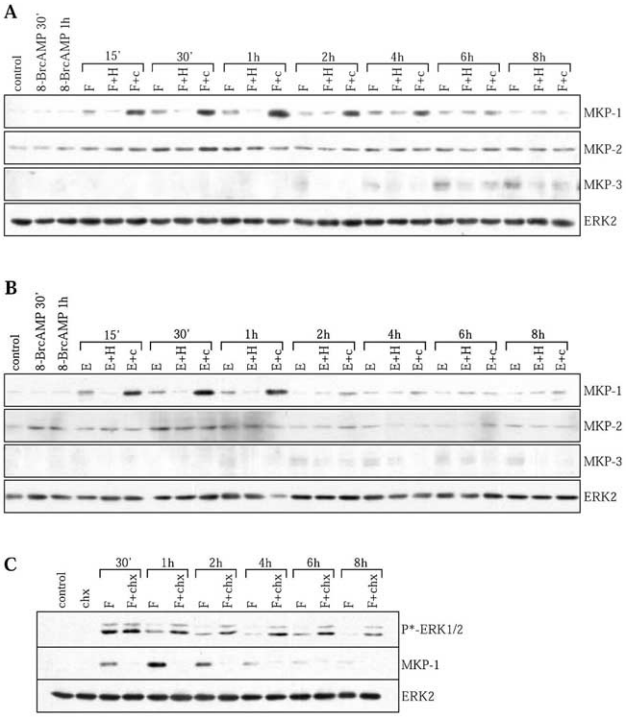

Fig. 4. PKA regulates growth factor-induced production of MKP-1 protein. Serum-starved cells were treated with H89 or 8-BrcAMP for 30 min prior to growth factor stimulation. The expression of MKPs was studied from total cell lysates by immunoblotting with antibodies against the different phosphatase species. Immunoblots with anti-MKP-1, anti-MKP-2 and anti-MKP-3 antibodies of lysates from (A) FGF-2- and (B) EGF-treated cells. (C) Serum-starved cells were treated with cycloheximide for 10 min (chx; 10 μg/ml) prior to FGF-2 stimulation. Phosphorylation of ERKs was studied by immunoblotting with anti-phospho-ERK1/2 antibodies. To correlate the expression level of MKP-1 to the amount of phosphorylated ERKs, the membranes were stripped and re-probed with anti-MKP-1 antibodies. The loading of the samples was studied by immunoblotting with anti-ERK2 antibodies. Each type of experiment was repeated three times with similar results.

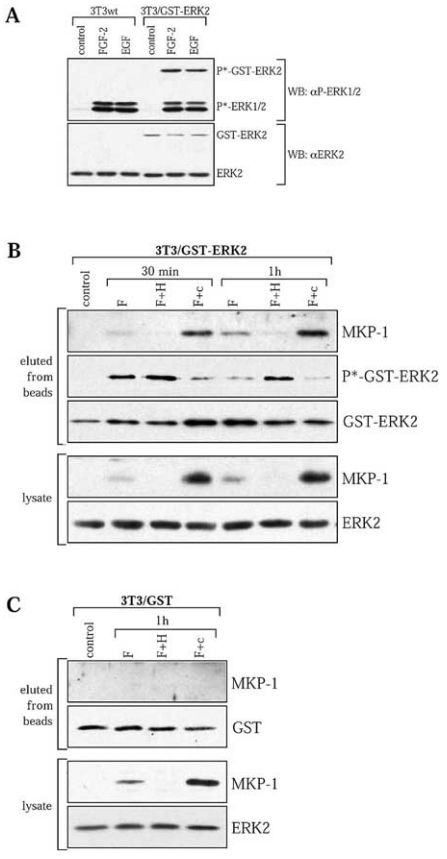

Fig. 5. PKA-mediated up-regulation of growth factor-induced MKP-1 expression increases complex formation between MKP-1 and ERK2. A: Immunoblots from wild-type and pEBG-GST-ERK2-transfected NIH3T3 cells showing the expression levels and growth factor-induced phosphorylation of the GST-ERK2 protein. B: To study the association of MKP-1 with ERK2 differentially treated cells were lysed in GST-pulldown buffer after which GST-ERK2 proteins were recovered using glutathione-Sepharose beads. The glutathione-Sepharose-bound GST-ERK2, along with associated proteins, was eluted using SDS buffer. The association of MKP-1 with GST-ERK2 was investigated with anti-MKP-1 immunoblots. The amount of GST-ERK2 protein in the samples was studied by anti-ERK2 immunoblot. The expression level of the MKP-1 protein was studied from cell lysates (30 μg of protein) by immunoblotting with anti-MKP-1 antibodies. C: Immunoblots from 3T3 cells stably expressing GST protein (3T3/GST), showing no association of MKP-1 protein with the GST protein. Each type of experiment was repeated three times with similar results.

To show that protein synthesis was required for the ERK dephosphorylation, cells were pre-treated with cycloheximide and analyzed for FGF-2-induced ERK phosphorylation by immunoblotting. The results (Fig. 4C) showed that cycloheximide abolished the FGF-2-induced expression of MKP-1 and consequently prevented the dephosphorylation of ERKs. These results demonstrate that PKA controls the growth factor-induced expression of MKP-1, and confirms that the dephosphorylation of ERKs requires growth factor-induced protein synthesis.

3.5. Up-regulated expression of MKP-1 enhances formation of MKP-1/ERK2 complexes and dephosphorylation of ERK2

To study whether the PKA-mediated regulation of MKP-1 expression would affect the complex formation between the activated ERKs and MKP-1, we established NIH3T3 cell lines

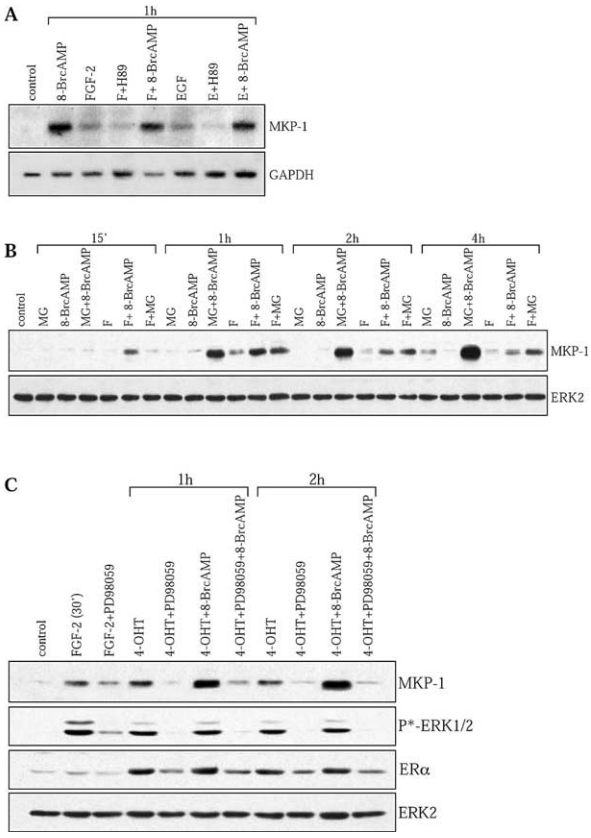

Fig. 6. PKA-mediated regulation of growth factor-induced expression of MKP-1. A: Serum-deprived cells were treated with 8-BrcAMP, H89 and growth factors alone or in combination followed by Northern analysis of MKP-1 and GAPDH mRNAs. B: Cells were treated with the proteasome inhibitor MG132 (10 μM) prior to PKA activation (8-BrcAMP) or growth factor inductions. Accumulation of MKP-1 was investigated with anti-MKP-1 immunoblots of total cell lysates. The loading was studied with anti-ERK2 immunoblot. C: Serum-starved NIH3T3 cells expressing the ΔRaf-1:ER[DD] fusion protein were pre-treated with 100 nM 4-OHT for 12 and 24 h followed by treatment with 8-BrcAMP (500 μM) for indicated times. To block the activation of Ras/ERK pathway PD98059 (20 μM) was introduced to the cells 30 min prior to FGF-2 induction or at the same time as 4-OHT. MKP-1 expression was studied by anti-MKP-1 immunoblots from total cell lysates. Several experiments were performed using both 12 and 24 h 4-OHT stimuli and the results from representative experiments are shown. To demonstrate that the ΔRaf-1:ER[DD] fusion protein was functional the membranes were stripped and blotted with antibodies against phosphorylated ERK1/2 and human estrogen receptor (ERα). The loading was studied with anti-ERK2 immunoblot. These experiments were repeated at least three times with similar results, representative experiments are shown.

with low constitutive expression of GST-ERK2 fusion protein (3T3/GST-ERK2) (Fig. 5A).

The interaction between MKP-1 and GST-ERK2 fusion protein was studied by GST-pulldown assays followed by immunoblotting with anti-MKP-1 antibodies. Activation of PKA prior to FGF-2 stimuli increased the expression of MKP-1 protein, and consequently the amount of GST-ERK2-bound MKP-1 and dephosphorylation of GST-ERK2, whereas inhibition of PKA activity abolished the expression as well as the binding and dephosphorylation (Fig. 5B). Notably, the complex formation between MKP-1 and GST-ERK2 correlated to the expression level of MKP-1. Pulldown assays with the cells producing only the GST part of the fusion protein (3T3/GST) did not yield MKP-1 protein, showing that the binding required ERK2 (Fig. 5C). These data suggest that, by regulating the growth factor induced expression of MKP-1, PKA would control the complex formation between ERKs and MKP-1 and subsequently the dephosphorylation of ERKs.

3.6. PKA and Ras/ERK pathways co-operatively regulate expression and stability of MKP-1 protein

We next investigated the mechanisms by which PKA and growth factors co-operatively regulate the expression of *mkp-1* gene and the production of MKP-1 protein. In agreement with previous data [50,51], activation of PKA by 8-BrcAMP clearly increased the transcriptional activity of *mkp-1* gene whereas growth factor stimulation had only a weak effect on the transcription. In cells treated with both 8-BrcAMP and growth factors, the levels of MKP-1 mRNA remained similar than in cells treated with 8-BrcAMP alone (Fig. 6A). Inhibition of PKA activity prior to growth factor treatment abolished the transcription, indicating that PKA is required for growth factor-mediated activation of the *mkp-1* gene. Activation of PKA alone did not induce production of MKP-1 protein, whereas the combined 8-BrcAMP/growth factor treatment significantly increased the amount of MKP-1 protein (Figs. 4A,B and 6B). These findings suggest that in addition to a weak transcriptional induction, the growth factor signals also regulate the stability of the MKP-1 protein. Brondello and co-workers recently reported that ERK-mediated phosphorylation of MKP-1 increased its stability by decreasing ubiquitin-directed proteolysis [33]. To investigate whether the absence of MKP-1 protein product upon PKA activation was due to rapid ubiquitin-mediated degradation we used MG132 to block the proteasome pathway. As shown in Fig. 6B, treatment of cells with 8-BrcAMP and MG132 led to a massive accumulation of MKP-1 protein. Our data thus suggest that PKA activation alone is sufficient to induce production of MKP-1 protein, but that the protein product is rapidly degraded by ubiquitin-directed proteolysis in the absence of growth factor-mediated stimulation of the Ras/ERK pathway to stabilize the protein.

To confirm that the accumulation of the MKP-1 protein in growth factor-stimulated cells required co-operative function of the PKA and Ras/ERK pathways, we established NIH3T3 cell lines with stable expression of a fusion protein where the ligand binding domain of human estrogen receptor was combined to the catalytic domain of Raf-1 (ΔRaf-1:ER[DD]). In this system the Ras/ERK pathway can be activated by 4-OHT without affecting other signaling pathways [65,66]. Importantly, although PKA has been shown to influence Raf-1 activity upon growth factor stimulation, it did not affect the 4-OHT-induced activity of the ΔRaf-1:ER[DD] fusion protein (data not shown). The 4-OHT-induced activation of the Ras/ERK pathway was rather slow, requiring prolonged 4-OHT induction to yield a level of ERK phosphorylation comparable to that obtained by growth factor stimulus. The results indicated that whereas 4-OHT alone had only a weak effect on MKP-1, concomitant activation of PKA significantly increased the accumulation of the MKP-1 protein (Fig. 6C). These data confirm the idea that besides PKA-induced transcription of *mkp-1* gene the growth factor-induced activation of the Ras/ERK pathway is required for the stabilization of the MKP-1 protein.

4. Discussion

Recent studies have demonstrated that increased PKA activity down-regulates the activity of the Ras/ERK pathway, suggesting a role for PKA in the regulation of growth factor signaling. However, important questions regarding the physiological significance of PKA in the regulation of the Ras/ERK signaling remain unanswered. In this paper we present data to support the idea that PKA has a central role in the growth factor signaling, and provide evidence for the important role of the basal PKA activity in such regulation. In these studies we have employed H89 to inhibit PKA activity. Although H89 is widely used to inhibit PKA signaling, we note that it may not be entirely PKA-specific since recent studies have shown its ability to down-regulate MSK1 and RSK2 [67,68]. In some situations the H89-mediated effects may be directed, at least partly, via these kinases but the results shown in this paper are clearly dependent on down-regulation of PKA activity.

We recently reported that in NIH3T3 cells the activation of PKA is regulated by FGF-2 and further that active PKA is required for the activation of growth factor-induced transcription and cell proliferation [54]. NIH3T3 cells display detectable basal PKA activity which may regulate the magnitude of growth factor signals, perhaps by maintaining a threshold which must be overcome before activation of signal transduction. When the threshold is overcome PKA would control the signal strength by down-regulating the Raf-1 activity and by increasing the MKP-1 expression, thus providing an effective mechanism to limit ERK activity. Our data thus point to a tight functional relationship between growth factor signaling and PKA.

Our results indicate that by blocking the PKA activity prior to growth factor stimulus Raf-1 activity was significantly increased, whereas PKA activation abolished the activation of Raf-1, conforming to the previous notion that PKA regulates the growth factor-induced activity of Raf-1 in a predominantly negative fashion. The regulation is thought to involve direct phosphorylation of Raf-1 by PKA [60–62,69]. However, the regulatory mechanisms are incompletely understood because Raf proteins contain several putative phosphorylation sites and are differentially regulated in different model systems [70]. PKA has been proposed to down-regulate PDGF-induced ERK activation by inducing MKP-1 [71]. Here we provide evidence that the Ras/ERK pathway and PKA co-operatively regulate the expression and function of MKP-1 protein and further that MKP-1 is critical for the PKA-mediated regulation of the Ras/ERK signaling. MKP-1 expression is induced by growth factors in a variety of cell types suggesting a negative feedback loop down-regulating the signaling through the Ras/ERK pathway [35–37,39,40]. The role of PKA in this loop may be to adjust and enhance the down-regulatory effect. Such mechanisms provide an effective way to rapidly dephosphorylate nuclear ERKs and limit the growth factor signaling close to its end point, in the nucleus.

How does PKA regulate the expression of *mkp-1* gene? The transcription of *mkp-1* gene is regulated by two CRE sites in its promoter region that work together with an E-box element [52]. PKA activation induces binding of CREB proteins to the CRE sites and forms, together with E-box-bound USF proteins, a complex able to promote transcription of the adjacent gene. This explains why PKA alone is sufficient to induce transcription of *mkp-1* gene. By contrast, growth factor stimulation appears a weak activator of the *mkp-1* gene (Fig. 6A). Kwak and co-workers showed that the basal promoter region does not mediate the growth factor signals since the 800 bp region containing the transcriptional initiation site responds poorly to FGF-2 or EGF [34]. Instead, the Ras/ERK pathway appears to be critical for the regulation of MKP-1 protein turnover, presumably involving phosphorylation of serine residues 359 and 364 preventing the ubiquitin-mediated degradation [33]. Our data suggest that even a weak signal via the Ras/ERK pathway after PKA-mediated down-regulation of Raf-1 activity is sufficient to stabilize the MKP-1 protein leading to efficient accumulation of MKP-1 and dephosphorylation of ERKs.

In summary, our data indicate that PKA may secure the growth factor-induced signaling by balancing the activity level of the Ras/ERK pathway. The balancing effect is mediated by regulating the activity of Raf-1 and the expression and stability of MKP-1. In particular, our results suggest an important role for the basal PKA activity in such regulation. The mechanism proposed may be involved in the maintenance of a physiologically relevant level of ERK activity, and moreover this PKA-mediated regulation might function as a back up mechanism to ensure that specific growth factors elicit proper responses in target cells.

Acknowledgements: We thank Dr. Michyuki Matsuda for the pEBG-ERK2 plasmid and Dr. Martin McMahon for the ΔRaf-1:ER[DD] plasmid. We are grateful to Susanna Pyökäri for technical help.

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